THE APPLICATION OF PAPER CHROMATOGRAPHY OF FLUORESCENT COMPOUNDS TO THE SYSTEMATICS OF FIREFLIES (COLEOPTERA, LAMPYRIDAE)

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Abstract

The fluorescent compounds of 13 species in 5 genera of fireflies have distinctive generic, specific, and population variation, as revealed by 2-dimensional paper chromatography. Spectrophotofluorometry of the fluorescent compounds was not useful for typifying the chemicals.

Introduction

The chemical constituents of organisms are potentially useful in taxonomy. Proteins, amino acids, and pigments have been used by previous workers (Leone 1964). Of interest here are the fluorescent chemicals, especially those comprising the largest group, the pteridines. Pteridines have been extensively studied biochemically and have been used in previous taxonomic work (Cockayne 1924; Rawson 1968; Waywell and Corey 1970). They were first discovered by Hopkins (1895) in the wing (hence the origin of the term) of a pierid butterfly, and were later named by Weiland and Schöpf (1925). The chemical structures of many have been determined, and others have been synthesized. Their biological role has been reviewed by Ziegler and Harmsen (1969). In insects they occur as metabolic end products and function as cofactors in hydroxylation reactions and as pigments. They are localized in the cuticle, wing scales, hypodermis, compound eyes, nervous system, light organ (of Lampyridae), and numerous other structures (Ziegler and Harmsen 1969; Table IV). The kinds and quantities of pteridines found in insect tissues vary with developmental stage (Ziegler and Harmsen 1969).

There are other fluorescing compounds detected by the same tests that are used for pteridines, and these may also have taxonomic value. These include tyrosine, phenylalanine, xanthine, hypoxanthine, uracil, and uric acid (J. Nation, personal communication, 1972).

Cockayne (1924) used a mercury vapor lamp to detect fluorescent compounds in intact specimens of Lepidoptera in 21 families. He found some families to have diagnostic fluorescent colors, and noted intra- and interspecific variation, as well as consistent sexual differences.

Rawson (1968), using paper chromatography on ground, whole specimens, found 19 different compounds in 13 species of Lepidoptera. He used 2 independent solvent systems, each producing a characteristic grouping of pteridines and other fluorescent compounds, and concluded that the occurrence of the fluorescent compounds correlated with confirmed morphological groupings. Rawson found fluorescent pigments to be of little use in separating groups larger than genera, except in the family Pieridae.

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Waywell and Corey (1970) did similar work with crayfish. They used 9 species from 2 genera. Five males and 5 females of each were homogenized and "run" on cellulose thin-layer chromatographic plates. Fifteen distinct spots were distinguished. Each species had its own chromatographic pattern, and the genera (*Cambarus* and *Orconectes*) could be distinguished by a single spot. Sexual differences occurred in only 1 of the 9 species. *Orconectes propinquus* and *O. rusticus* differed by only 1 spot, thus supporting the morphological evidence of field-collected intermediate hybrids that these 2 are closely related. Hybridization between *O. propinquus* and *O. obscurus* had been suggested, but, since they differed by 3 spots, Waywell and Corey concluded hybridization was probably not occurring. The presence of a single band consistently separated 2 other species, whereas morphological characters did not.

METHODS AND MATERIALS

Fireflies used in this study were collected in Gainesville, Hines, and Cedar Key, Florida; Athens, Tennessee; Ann Arbor and Pellston, Michigan; and Oneida, New York. Specimens were held in pint jars containing damp vegetation and slices of apple for not less than 24 hours. Males were used, since only they could be identified with certainty.

Specimens to be analyzed were washed individually in distilled water and macerated with a Pyrex 16 × 150mm No. 7725 tissue grinder in sufficient distilled water to yield 1-1.5ml homogenate.

Each specimen was processed individually using 2-dimensional chromatography. Approximately 0.5ml of homogenate from a single specimen was placed 2.5cm from each margin in a corner of a 20 × 20cm sheet of Whatman No. 3 chromatography paper (the resulting spot was 1.2 to 2.5cm in diameter). The paper was then rolled into a cylinder and fastened with staples without allowing the abutting margins to come together. Before treatment with the second solvent system the sheet was dried at least 12 hours in room conditions. The solvent systems used were 1) 1-propanol, ammonium hydroxide and water (in proportions 8:3:1) and 2) butanol, acetic acid, and water (12:3:5). Large-mouth gallon jars were used for chromatographic chambers. They were covered with glass plates and sealed with stopcock grease to maintain a saturated atmosphere. Sufficient solvent to cover 1.2cm of the chromatogram was maintained. Solvents were renewed weekly or after about 6 separations. Chromatograms were left in the solvent systems approximately 4 hours or until the solvent front had moved to about 1.2cm from the top of the paper. Temperature during separation was $25 \pm 3°$ C. Chromatograms were exposed to normal room lighting throughout the procedure but not to direct sunlight.

The chromatograms were read, after drying at room temperature with a UVSL multi-band Mineralight® ultraviolet lamp with wavelengths of 254 mμ and 365 mμ. Fluorescent areas were circled with pencil, and a subjective evaluation of the color recorded. R_f values were calculated for each migrated fluorescent area.

The same chromatographic procedures were performed on known compounds: xanthopterin (0.5 μg), pterin-6-carboxic acid (0.5 μg), 2-amino-6, 7-dimethyl-4-hydrox pteridine (1 μg), xanthine (2 μg), hypoxanthine (2 μg), and uric acid (2 μg). This gave an indication of R_f value variability.

To further typify the fluorescent spots, excitation and emission spectra were determined for a total of 320 knowns and unknowns using an Aminco-Bowman spectrophotofluorometer. Spectral analyses were not consistent in the unknowns tested. Spectra of known compounds were consistent and repeatable only if concentrations were high enough to overcome the effects of scatter and extraneous fluorescence. Spectrophotofluorometric analysis is a potentially useful tool in work of this type, but was not effective in this study (See Wilkerson 1973, for details).

Results

The first step in compound recognition was to compare all the chromatograms of a species. All spots of like color occurring in nearly the same position were used for the study.[2] Some spots were diffuse or small, but consistency dictated their use.

Chromatogram summary graphs were then made for each species by tracing all consistent spots on onion-skin paper. Graphs were compared in order to recognize overlap of compounds of like color and similar position. The same compound number could then be assigned to compounds in different species. Some compounds not overlapping in R_f value range but nevertheless spatially close were subjectively placed in the same compound group. This was done since the possible range of R_f overlap was not statistically determined.

The following is an evaluation of the 51 spots which were recognized. Graphic representation of all spots is found in the Figures. Table 1 contains color evaluations of the compounds.

No precise evaluations of relative intensity or size were made; thus the following descriptions of single compounds or groups of compounds are subjective.

Generic Differences:

Spots which appear exclusively in a given genus range from 8% (*Micronaspis*) to 58% (*Pyropyga*) of the total spots in that genus. Only 3 of these unique spots are common to all species within a genus, 1 each in *Photuris, Micronaspis,* and *Photinus* (compounds 18, 37, and 2 respectively).

Photuris (5 species examined).

Compound 18 in *Photuris* is shared by all 5 species. It is found, however, in a relatively low percentage of specimens (40-70%), is of pale intensity, and is not easily detected. The other compounds unique to the genus (19, 21, 22, 16) are also found in low percentages (31-57%).

Pyractomena (2 species examined).

Spots unique to this genus (26, 33, 35, 36, 50) are nearly always present and easy to recognize. However, none of these are common to both species.

Micronaspis (monotypic genus).

Micronaspis floridana Green has an easily seen, unique spot (37) in 100% of the specimens.

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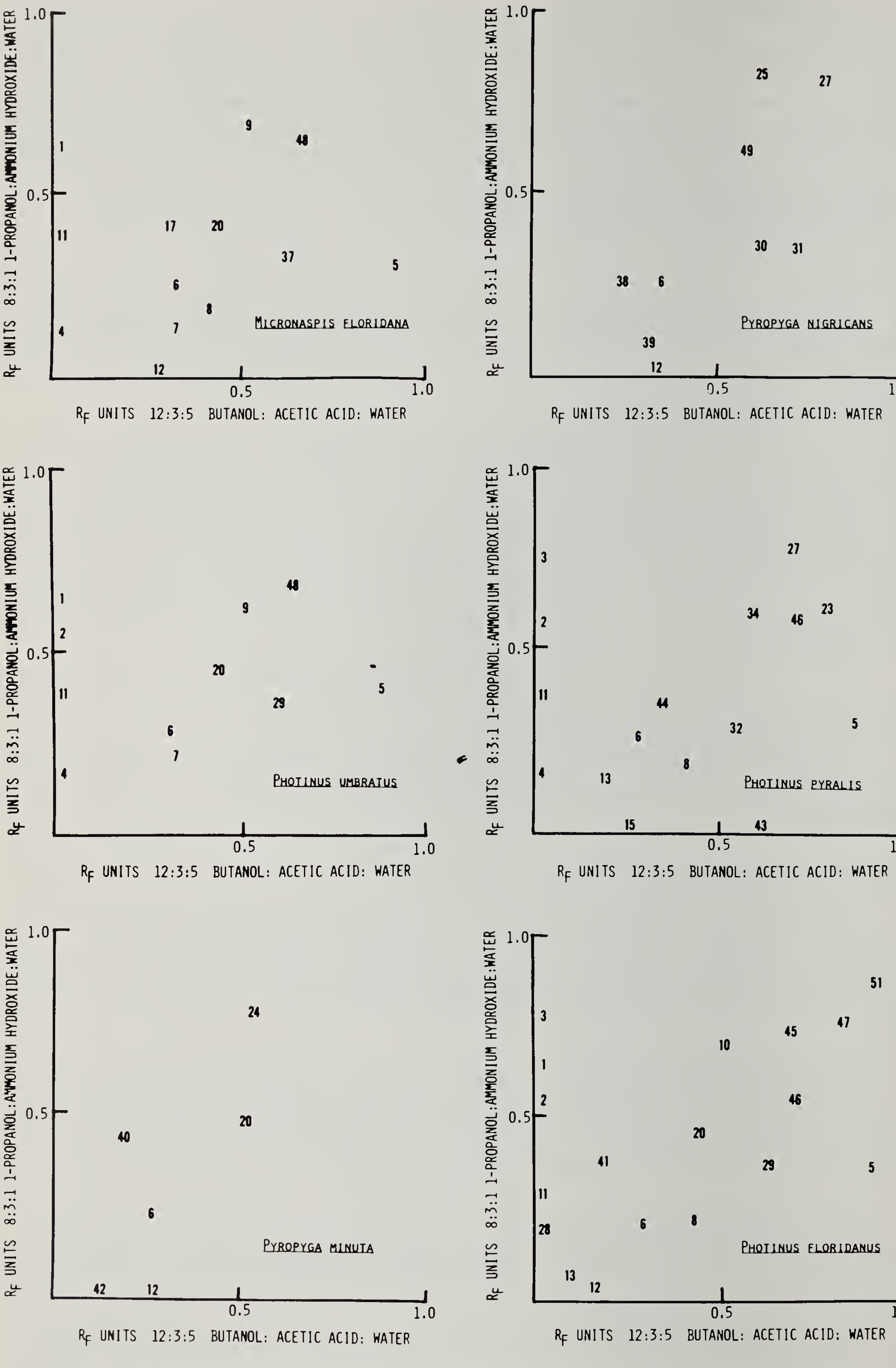
[2] Ranges of R_f values and percent occurrence in each species are given in Wilkerson, 1973.



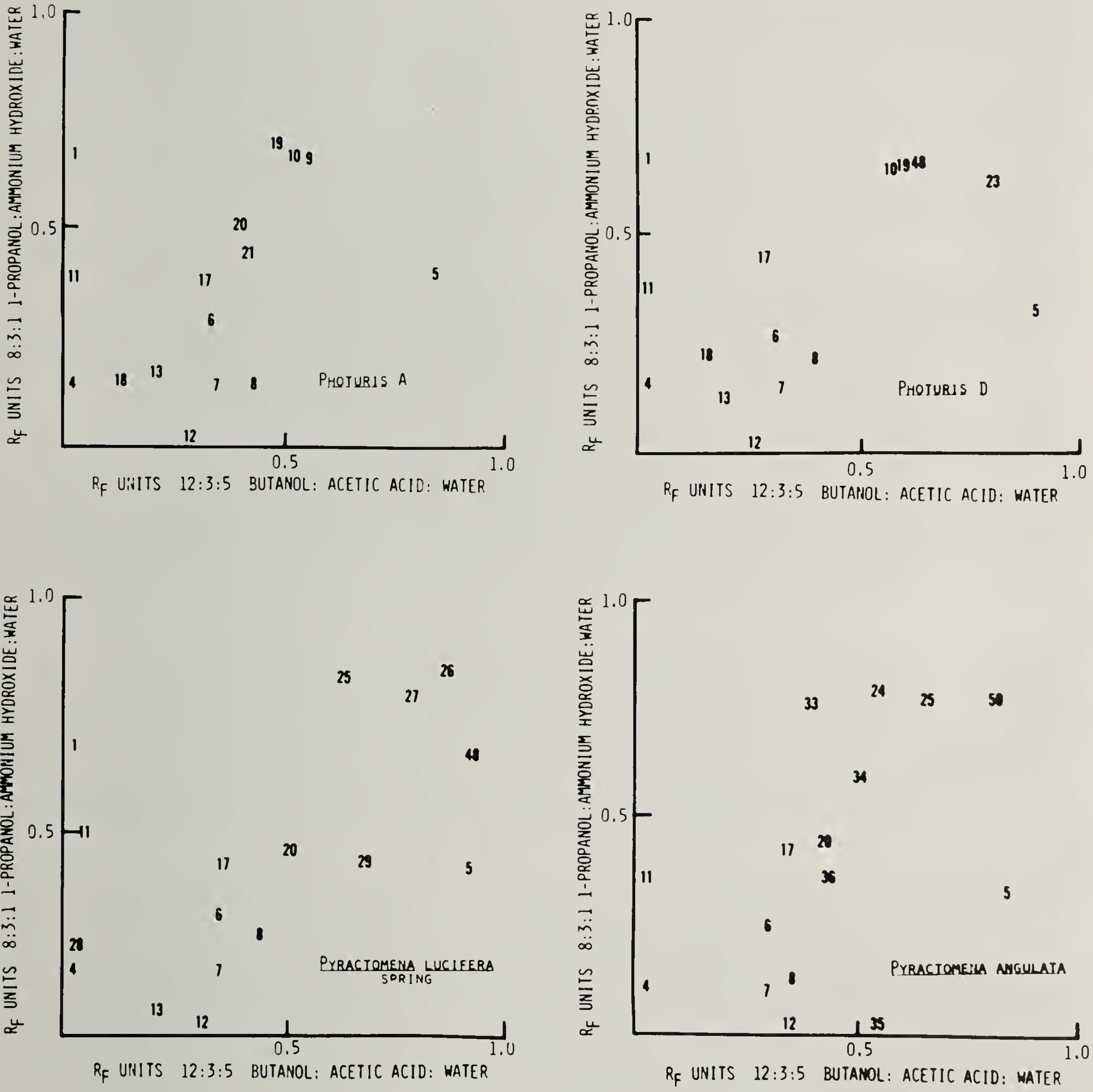
Graphic representation of R_f values for fluorescent compounds of various species of fireflies. Each graph represents a composite of all specimens processed for that species. Compounds were recognized on the basis of R_f value and color.

Pyropyga (2 species examined).

There are no spots common to both species yet absent in other genera, although 7 of the 13 spots present in the 2 species are found in no other genus (30, 31, 38, 39, 40, 42, 49). This is the highest percentage (58%) of unique spots in a genus. These spots are generally easily recognized (except 38 and 39 which are difficult to see) and occur in fairly high percentages (100, 100, 50, 75, 67, 100, and 80% respectively).

Photinus (3 species examined).

Ten of the 29 total spots in *Photinus* (2, 3, 15, 32, 41, 43, 44, 45, 46, 47) are found only in this genus, and numbers 2, 5, and 11 are common to all species within the genus. All of the above spots are distinct and easily recognizable except number 15.



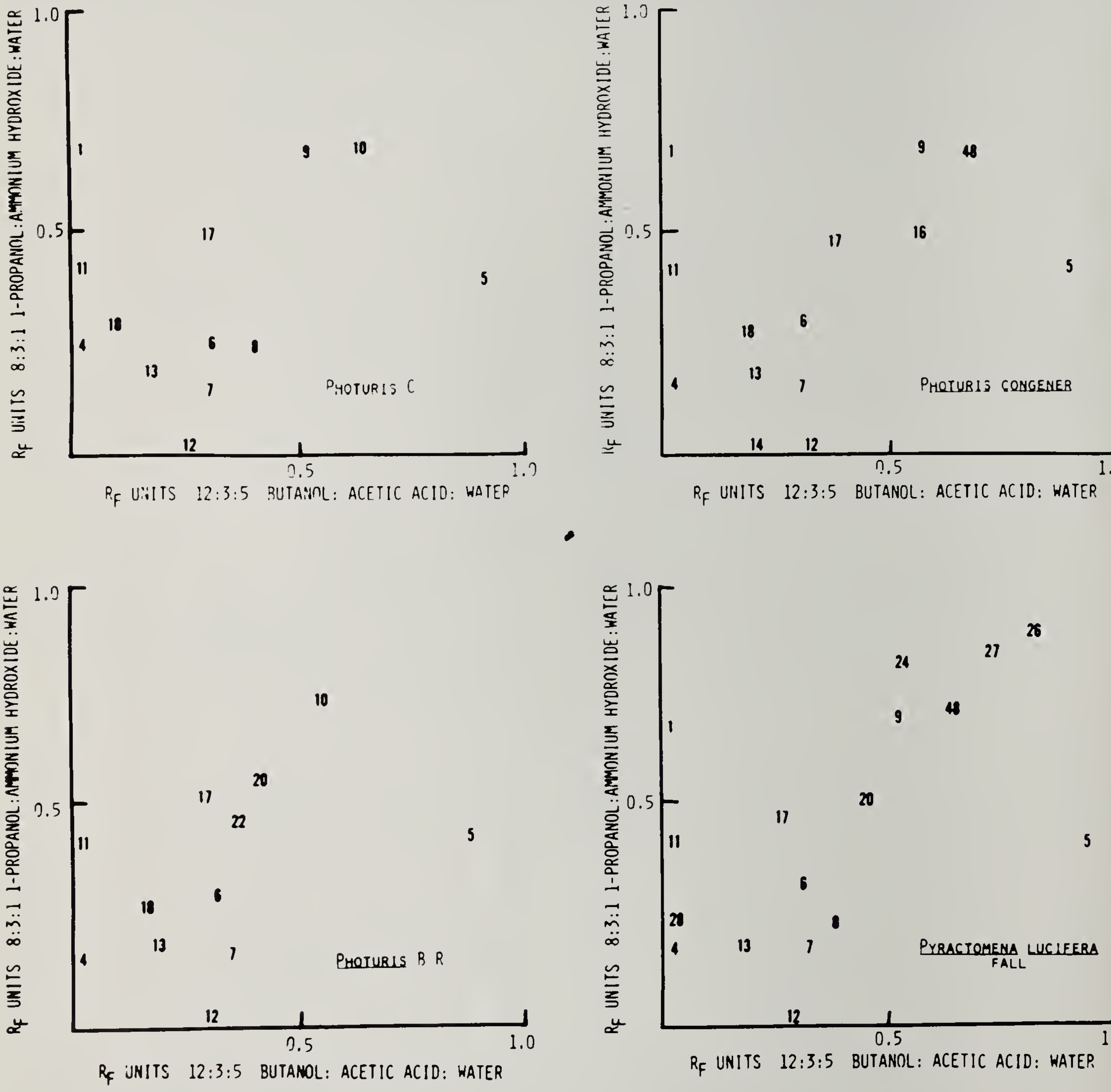
Graphic representation of R_f values for fluorescent compounds of various species of fireflies. Each graph represents a composite of all specimens processed for that species. Compounds were recognized on the basis of R_f value and color.

Species Differences:

No 2 species exhibited identical combinations of fluorescent compounds. Since allochronic populations of *Pyractomena lucifera* exhibited some differences, the reliability of this test as a basis for species separation must be considered.

Photuris[3]

Many fluorescent compounds were common to all 5 species. Nine of 21 spots (43%) are alike in all species indicating a high degree of homogeneity



Graphic representation of R_f values for fluorescent compounds of various species of fireflies. Each graph represents a composite of all specimens processed for that species. Compounds were recognized on the basis of R_f value and color.

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[3] The species designated "C", "A", "D", and "BR" in the genus *Photuris* are undescribed and will be assigned binomens later by JEL.

within the genus. Good species differences are seen in a group of compounds (9, 10, 19, 48) found in various combinations.

Photuris "C" (n = 5) has compounds 9 and 10 (compounds 19 and 48 are absent).

Photuris congener Le Conte (n = 6) has numbers 9 and 48 plus 2 spots (14, 16) found in none of the other 12 species.

Photuris "A" (n = 13) has 9, 10, and 19 forming a rather complicated grouping separated by color (9-green, 10-blue and light, 19-purple). Compound 21 is unique to this species.

Photuris "D" (n = 7) has 9, 10, and 48. Spot 23 is found in only 1 other species (*Photinus pyralis*).

Photuris "BR" (n = 6) has compounds 10 and 22. Compound 22 is unique to it.

Pyractomena.

Nine of 24 (38%) of the spots are common to all species. This shows a relatively high degree of homogeneity.

Pyractomena lucifera (Melsheimer) was studied in 2 groups; 1 from a spring population, the other from a fall population collected at the same locality. For purposes of analysis the 2 were treated as different "species".

P. angulata (Say) (n = 5) is distinguished by a pattern of spots (33, 24, 25, 50, 34). Three spots are unique; 35 and 36 are found in all specimens, and 50 in 40% of the specimens.

P. lucifera (spring) (n = 9) is recognized by a pattern consisting of compounds 29, 48, 25, 27, and 26. Spot 29 is unique to *P. lucifera* within the genus but is also found in 2 *Photinus* species.

P. lucifera (fall) (n = 5) has a pattern similar to the spring group if the chromatograms are viewed subjectively but the R_f values of spot 25 (spring) do not correspond with spot 24 (both are yellow) found in nearly the same position in the fall group. In the absence of spectral data, we therefore assumed these to be different chemicals. In addition, spot 9 is present only in the fall group and spot 29 only in the spring group. This produces a 24, 27, 26, 9, 48 combination.

These 2 groups of *P. lucifera* are similar except for the 4 spots (9, 29, 24, 25) mentioned above. Compound 26 was found in all specimens of both populations and is therefore an excellent indicator; number 28 was found in only 20% of the fall and 44% of the spring individuals, and elsewhere only in *Photinus floridanus.* Compound 28 would seem less reliable because of incidence in fewer specimens, but when present it is distinct.

Photinus.

Photinus has only 4 of 29 total spots (14%) which are common to all 3 species studied; the 3 species belong to 3 different species groups.

Photinus umbratus Le Conte (n = 11) has 2 spots (9, 48) which are unique to it within the genus. Spot 9 is found only in 27% of the specimens. There are no spots occurring only in this species.

P. pyralis (Linnaeus) (n = 7) has a sequence of easily-seen spots (34, 46, 23, 27) and 4 spots (15, 32, 43, 44) which are unique.

P. floridanus Fall (n = 5) has a sequence of spots (46, 10, 45, 47, 51) indicative of this species and 4 (41, 45, 47, 51) found in no other species.

Paper Chromatography With Knowns:

R_f value variation around the means of both known and unknowns is ±0.05 R_f units or more. F tests (Mendenhall 1971:245) comparing 5 selected sets of R_f values of unknowns to the 10 sets of R_f values from known compounds revealed only 4 of the 50 tests as showing variation in the unknown compound greater than in the known. This shows variation caused by contamination of whole processed insects is probably of little importance in introducing error.

Uric acid was difficult to detect in first-direction separation and could not be detected at all in second-direction separation.

Xanthopterin and 2-amino-6, 7-dimethyl-4-hydroxypteridine left a trace of fluorescence at the origin of second-direction separation. The cause of this "ghost" is not known. It is suspected that some of the unknown compounds behaved in this way also since spots remained at the origin in several species (compounds 1, 4, 11). It is not definitely known if in fact these spots are the result of the "ghost" effect.

Color changes were noted after separation in the 2 solvent systems for xanthopterin and pterin-6-carboxylic acid. This may be chemical interaction with the solvents or a function of the pH of the 2 systems.

DISCUSSION

The chromatographic methods used in these experiments can be improved in a number of ways to produce a better systematic tool. Many of the problems encountered in chromatography are discussed by Heftmann (1967).

We encountered 2 major problems, R_f value variation (±0.05 R_f units or more) and spreading of the developed spot. Variation of R_f values and spot spreading may be controlled by choice of solvent systems. Only those compounds with R_f values between 0.12 and 0.90 R_f units are considered reliable (Heftmann, 1967). Since each solvent will affect each compound

TABLE 1. FLUORESCENT COMPOUND COLOR EVALUATION.

Color	Compound Number
Blue	1, 2, 3, 4, 5, 7, 10, 16, 17, 20, 23, 26, 27, 29, 30, 31, 32, 34, 36, 38, 40, 41, 43, 44, 45, 46, 47, 48, 49, 51
Dark Blue	19
Light Blue	1, 9, 10, 17, 23, 26, 48
Blue-Green	1, 9, 17, 26, 28, 39, 48
Purple	6, 12, 13, 21, 22, 42, 19
Green	1, 8, 9
Yellow-Green	1, 8, 28
Yellow	1, 5, 8, 11, 14, 15, 18, 24, 25, 28, 33, 35, 37, 39, 50
Pink	5

In a few cases spot size, position, intensity, shape and/or consistency indicated that a single compound was involved, yet there was considerable color variation (e.g. no. 1; see figures). A number of causes seem possible, and we chose to assign a single number.

differently, it may be possible to find solvents to place all spots within the proper range.

Experimental conditions which are rigidly controlled will also give more consistent results. Heftmann (1967) listed factors that are applicable to this study.

In conjunction with the above it may be desirable to increase the sensitivity (resolving power) of the test. Whatman No. 1 (or a comparable paper) gives much better resolution than the thicker Whatman No. 3. Thin-layer chromatography is 10-100 times more sensitive than paper. There are 2 parameters not utilized in this work which could give useful data. These are spot size measurement and spot brightness. Spot brightness could be compared to standard concentrations of known fluorescent compounds.

The limits of predictive capabilities for fluorescent compound analysis as a systematic tool must be investigated. These limits can be tested by answering the following questions: 1) What sample size will be required for good statistical analysis? 2) What is the range of variation in the numbers of possible compounds and their frequency of occurrence within a population? This range is necessary to compare species. 3) What variations exist among populations collected from different points in a species' range? 4) What effect does specimen age have on the test? Laboratory rearing may be required for definite age determination. 5) Does larval or adult diet affect the spots found? 6) Will gut content affect the occurrence of spots? If the answer to this question is affirmative, it may also be asked, will it be possible to determine what species of firefly a female *Photuris* aggressive mimic (Lloyd 1965) has recently eaten? 7) Do differences occur in temporally separated populations? 8) Is it possible to use dried or preserved specimens? The work by Cockayne (1968), reviewed earlier, suggested this may be possible for dried specimens. Schmidt (1969) separated 38 fluorescent compounds from *Formica* ants preserved in 70% ethanol, suggesting alcohol preservation may be possible also. 9) Can parts of a specimen be used? Also which (e.g., leg, light organ, head) will give the most information? This is important to know if valuable museum specimens are used. 10) What sex differences exist?

Conclusions

Paper chromatography of fluorescent compounds could be an effective means of generic and specific determination in the family Lampyridae. Refinements of technique and more comprehensive sampling are needed to determine the full potential of this procedure as a systematic tool in fireflies and in other insect groups.

The need for new parameters in insect systematics is great. The use of fluorescent compounds as systematic characters holds much promise as a new and relatively unexplored means to aid in the complex process of insect classification and identification.

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